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Enzyme and Microbial Technology 36 (2005) 832–839

ENZYME and MICROBIAL TECHNOLOGY

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Metabolic engineering of *Torulopsis glabrata* for improved pyruvate production

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Received 13 April 2004; accepted 10 January 2005

Abstract

During pyruvate production, ethanol is produced as a by-product, which both decreases the amount of pyruvate and makes the recovery of pyruvate more difficult. Pyruvate decarboxylase (PDC, EC 4.1.1.1), which degrades pyruvate to acetaldehyde and ultimately to ethanol, is a key enzyme in the pyruvate metabolism of yeast. Therefore, to order to increase the yield of pyruvate in *Torulopsis glabrata*, targeted *PDC*-disrupted strains were metabolically engineered. First, *T. glabrata ura3* strains that were suitable for genetic transformation were isolated and identified through ethyl methansulfonate mutagenesis, 5-fluoroortic acid media selection, and *Sacchramyces cerevisiae URA3* complement. Next, the *PDC* gene in *T. glabrata* was specifically disrupted through homologous recombinant with the *S. cerevisiae URA3* gene as the selective marker. The PDC activity of the disruptants was about 33% that of the parent strain. Targeted *PDC* gene disruption in *T. glabrata* was also confirmed by PCR amplification and sequencing of the *PDC* gene and its mutants, PDC activity staining, and PDC Western blot. The disruptants displayed higher pyruvate accumulation and less ethanol production. Under basal fermentation conditions (see Section 2), the disruptants accumulated about 20 g/L of pyruvate with 4.6 g/L of ethanol, whereas the parental strain (*T. glabrata* IFO005) only accumulated 7–8 g/L of pyruvate with 7.4 g/L of ethanol. Under favorable conditions in jar fermentation, the disruptants accumulated 82.2 g/L of pyruvate in 52 h.

Keywords: Pyruvate; Pyruvate decarboxylase; *ura3* Auxotroph; Gene disruption; *Torulopsis glabrata*; Ethanol; Metabolic engineering

1. Introduction

Pyruvate is a key metabolite in the glycolytic pathway; it is also a model product for studying the regulation of glycolysis [1]. Pyruvate is the key substrate for the enzymatic production of amino acids such as L-tryptophan, L-tyrosine, and L-dihydroxyphenylalanine (L-DOPA) [2,3]. The commercial demand for pyruvate has been increasing due to its use as a starting material for the synthesis of many drugs and agrochemicals, and as a component of animal cell cultures. Pyruvate also has a healthcare function (i.e., it has been recently approved as a dietary supplement) [4].

Pyruvate is associated with many metabolic pathways in microorganisms and it is not usually accumulated or secreted at any significant level by microorganisms. However, under certain conditions excess pyruvate production has been observed from bacteria [5], yeasts [6], molds, and basidiomycetes [7]. With the advent of various modern molecular biotechniques, many higher-pyruvate-producing strains were successfully engineered [8–10]. Optimum process control strategies have also made great contributions to enhancing pyruvate production [11,12].

In order to get a high yield and increase the productivity, it is necessary to accelerate glycolysis and/or to inhibit the activities of the enzymes that are responsible for pyruvate degradation in yeasts [13]. Generally, three key enzymes-pyruvate decarboxylase (PDC), pyruvate dehydrogenase complex (PDH complex), and pyruvate carboxylase (PYC) are responsible for pyruvate catabolism in yeasts [1]. These enzymes all need vitamins or their derivatives as the coenzymes. Improving cultivation conditions, especially the concentrations

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0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.01.015

of required vitamins, was a possible way to control enzyme activity and direct the metabolism of yeast to accumulate high levels of pyruvate [6,14]. Using vitamin limitation as a strategy for metabolic control would be efficient but not perfect for pyruvate production because it would require many tricks; this is due to feeding different species and concentrations of vitamins in the process of fermentation. Other process strategies, such as oxygen supply control [11], also have some tricks. Treating wild-type pyruvate-producing yeast, *T. glabrata* IFO005, with chemical mutagens can generate mutants with reduced activity of pyruvate decarboxylase; thus increasing pyruvate production and decreasing ethanol production [15]. However, the genetic and metabolic profiles of mutants derived from chemical mutagenesis were poorly characterized and mutagenesis remained a random process where science was complemented with elements of art.

The aim of metabolic engineering is defined as the purposeful modification of metabolic networks in living cells to produce desirable chemicals with superior yields and productivity by using recombinant DNA technologies [16]. First suggested by Dr. Bailey in 1991, metabolic engineering powered by techniques from applied molecular biology and reaction engineering has since become very popular in the last decade. Major efforts have been made in selecting the appropriate microorganisms to both efficiently and abundantly produce chemicals of commercial interest. The notion of metabolic engineering significantly extends the range of microbes utilizable as productive hosts. In order to achieve these objectives, three main research approaches are usually employed: (1) introducing exogenous genes which convert the final precursor of a host organism to a desirable chemical at a viable yield; (2) enhancing metabolic flux through a pathway to increase the synthesis of the final precursor or product; and (3) minimizing the biosyntheses of by-products (or other products). There have been great changes in strain improvement since the dictionary of metabolic engineering.

In this particular, the third research approach of metabolic engineering (3) was applied to specifically disrupt the *PDC* gene in a pyruvate-producing yeast *T. glabrata*. As a result, the carbon flux was redirected in the yeast strain from ethanol formation with respect to increased production of pyruvate. Also, this study shows how PDC influences the pyruvate yield in *T. glabrat*. The resultant strains with higher pyruvate productivities and commercial potentials were also described and discussed.

2. Material and methods

2.1. Strains, plasmids, and culture conditions

Table 1 lists each strain and plasmid used for this study. *Escherichia coli* DH5α was used for plasmid construction, and *T. glabrata* IFO005 (a gift from Dr. Liu Jiquan, South Yangtze University, P.R. China) was used as the recipient strain. Recombinant *E. coli* strains were grown at 37 °C in Luria-Bertani medium [17] supplemented with ampicillin (100 mg/L). *T. glabrata* was grown in YEPD (10 g yeast extract, 20 g tryptone, 20 g glucose, pH 5.5) at 30 °C, 5-fluoroorotic acid (5-FOA) medium [18] or synthetic medium (SD) [19] except for pyruvate production.

Table 1
Stains and plasmids

Strains or plasmid	Related characteristic	Source or reference
Strains		
E. coli DH5α	*supE44ΔlacU169(φ80lacZΔM15) hsdR17recA1endA1gyrA96thi-1relA1*	Stratagene
T. glabrata		
IFO005	Wild type	Dr. Liu Jiquan
IFO005-3	*ura3*	This work
IFO005-7	*ura3*	This work
IFO005-36	*ura3 pdc::URA3*	This work
IFO005-72	*ura3 pdc::URA3*	This work
Plasmids		
pET21a(+)		Navogen
pRUL129		Dr. H. Yde Steensma
pETP1		This work
pEPU1		This work

2.2. Media for pyruvate production and its fermentation conditions

The seed medium with a pH of 5.5 was composed of 10% glucose, 3% fish peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 4 mg/L of nicotinic acid (NA), 200 μg/L of pyridoxine, 2 μg/L of biotin and 4% $CaCO_3$. The basal fermentation medium with a pH of 5.5 was composed of 10% glucose, 0.1% soybean peptone, 0.6% $(NH_4)_2SO_4$, 0.1% KH_2PO_4,0.05% $MgSO_4 \cdot 7H_2O$, 8 mg/L of NA, 1 mg/L of pyridoxine, 30 μg/L of biotin, 30 μg/L of thiamine and 4% $CaCO_3$. The yeast was cultivated in Erlenmeyer flasks at 30 °C at 200 rpm/min for 24–36 h. The jar fermentation medium with a pH of 5.5 was composed of 15% glucose, 3% fish peptone, 0.1% KH_2PO_4,0.05% $MgSO_4 \cdot 7H_2O$, 4 mg/L of NA, 0.1 mg/L of pyridoxine, 10 (g/L of biotin, 10 μg/L of thiamine, pH was maintained by supplementing of 5 N NaOH, agitation was 500 rpm and air supply was 1:1. The inoculum was 10% of the total volume of the medium.

2.3. Isolation and characterization of T. glabrata ura3 strains

Ethyl methansulfonate (EMS, Sigma Chemical Co., St. Louis, Mo.) mutagenesis of *T. glabrata* was performed as described for *Saccharomyces cerevisiae* [19]. Unless stated otherwise, EMS treatment killed approximately 50% of the initial population. *ura* (uracil auxotrophic phenotype) mutants were obtained on 5-FOA agars (*ura* mutants can grow on 5-FOA mediums, whereas others cannot). Plasmid pRUL129

Table 2
Oligonucleotide primers used in this study

Oligonucleotide primer	DNA sequence
PDC-C11	5′-GCGGATCCATGTCTGAAATTACTTTGG-3′
PDC-C12	5′-GTTCTCGAGCTTATTGCTTAGCGTTGG-3′
ScURA3-C1	5′-AGCAAGCTTTGAGAGTGCACCATAC-3′
ScURA3-C2	5′-GTTGGTACCTCTCCTTACGCATCTG-3′
TgURA3-C1	5′-CTTACAATGTCCAGTGCC-3′
TgURA3-C2	5′-TTGGATATGTCTTGAATCAG-3′
PDC-C21	5′-ATGTCTGAAATTACTTTGGT-3′
PDC-C22	5′-TTATTGCTTAGCGTTGGTGGAA-3′
PDC-C31	5′-GAGACCAGACTAATACAACT-3′
PDC-C32	Same as PDC-C12

[20] was transformed into *ura* mutants by modified lithium acetate method [19], and *ura3* mutants that could be complemented by the *S. cerevisiae URA3* gene on the plasmid pRUL129 were confirmed.

2.4. Construction plasmids and strains

The primers used in this study are shown in Table 2. The *T. glabrata PDC* gene (accession number AF545432) was amplified using primers PDC-C11 (forward) and PDC-C12 (reverse) with the genomic DNA of *T. glabrata* as the template so that a 1710-bp DNA fragment (Fragment I, Fig. 1A) containing a 5′*Bam*HI and 3′ *Xho*I site (underlining in the primers) was obtained. The genomic DNA of *T. glabrata* was prepared by the glass-beads method [19]. Fragment I was digested with *Bam*HI and *Xho*I to obtain an about 1.7-kb DNA fragment containing *T. glabrata PDC* coding region. This DNA fragment was then cloned directly into pET21a(+) to produce plasmid pETP1. The *S. cerevisiae URA3* gene was amplified using primers URA3-C1 (forward) and URA3-C2 (reverse) with plasmid pRUL129 as a template so that a 1182-bp DNA fragment (Fragment II, Fig. 1A) containing a 5′ *Hin*dIII and 3′ *Kpn*I site (underlining in the primers) was obtained. Plasmid pETP1 and Fragment II were digested with *Hin*dIII and *Kpn*I to obtain a 6470 and 1176-bp fragment, respectively. These two DNA fragments was ligated with T4 DNA ligase to produce plasmid pEPU1, which contained an about 2250-bp DNA fragment (Fragment III, Fig. 1A) including *T. glabrata PDC-F1, S. cerevisiae URA3*, and *T. glabrata PDC-F2*. *PDC-F1* and *PDC-F2* were located in the region of nucleotide 1–546 and 1170–1692, respectively, in *T. glabrata PDC* coding region. Fragment III was amplified using primers PDC-C21 (forward) and PDC-C22 (reverse) with plasmid pEPU1 as a template, and was transformed into *T. glabrata ura3* stains (IFO005-3 and IFO005-7) by the modified lithium acetate method. In vivo, fragment III replaced *PDC* gene coding region (1,692-bp) of *T. glabrata* IFO005-3 and IFO005-7 by homologous recombination, and *PDC* gene-disrupted *T. glabrata* strains IFO005-36 and IFO005-72 were constructed.

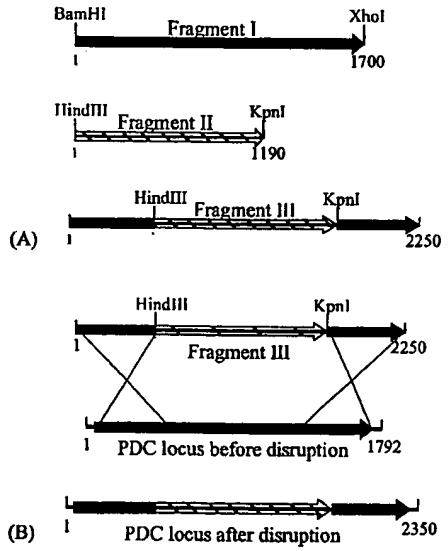

Fig. 1. Schematic representation of the DNA fragments for gene disruption and the disruption of *PDC* by homologous recombinant. *T. galbrata PDC* or fragments (black), *S. cerevisiae URA3* gene (*ScURA3*, hatched). (A) Structure of DNA fragments I–III. Fragment I denoted PCR product of *T. galbrata PDC*; Fragment II denoted PCR product of *S. cerevisiae URA3*; Fragment III denoted PCR product that containing *T. glabrata PDC-F1*, *S. cerevisiae URA3* and *T. glabrata PDC-F2* (from left to right); (B) *PDC* gene disruption by homologous recombination.

2.5. Analytical procedures

The fermentation broth was centrifuged at 10,000 × *g* for 20 min and the supernatant was used to determine metabolites. Pyruvate in the culture medium was determined by the DNP (2, 4-dinitrophenylhydrazine) method [21] with sodium pyruvate as standard. Glucose was determined by the DNS (3, 5-dinitrosalicylate) method [22]. Glycerol was determined by the Nash method [23]. Ethanol was determined by HPLC with a refractive index (RI) detector and an Aminex HPX-87H column (Bio Rad Laboratories, USA); the mobile phase was a 5 mM H_2SO_4 aqueous solution with a flow rate of 0.6 mL/min; the injection volume was 25 μL. Pyruvate, glycerol, and glucose concentration were also determined by HPLC under the same conditions. Yeast cell growth (biomass) was measured turbidimetrically at 660 nm (OD_{660}) after the culture broths were diluted 50-fold with water. The optical density value was converted to dry cell weight (DCW) using the calibration equation (1 OD_{660} = 0.3 g DCW/L).

2.6. Preparation of cell extracts and enzyme assays

T. glabrata IFO005 or its mutants were cultured aerobically at 30 °C for 16 h in 18 mm-ϕ tube containing 5 mL of YEPD or YEPD supplemented with 20 mg/L of uracil. Cells collected from the two media were washed with 20 mM sodium phosphate buffer (pH 6.0) and re-suspended in the same buffer. After being vertoxed with glass beads for 10 min at 4 °C, the cell extracts were centrifuged at 15,000 × *g* for 30 min to remove the glass beads and cell debris; PDC ac-

tivity in the supernatant was measured by a coupled enzyme assay [24] with a minor modification. Assays were performed at 30 °C and pH 6.0 in 100 mM citrate buffer, 33 mM sodium pyruvate with 1.5 units of yeast alcohol dehydrogenase (ADH) in 3 mL volume, unless otherwise noted. Enzyme activity was measured for the first 1–3 min.

2.7. Enzyme activity staining and Western blot analysis

Cell extracts were subjected to the native gradient PAGE using a 5–12.5% linear gradient slab gel with a running buffer consisting of 25 mM Tris and 192 mM glycine (pH 8.8) at 4 °C. Activity staining with 1,2-dianilinoethane [25] was utilized for the native gel. The protein extracts prepared for the enzyme activity assays were used in the Western blot analysis. Western blotting was performed as described [26] with some modifications and stained with dissolved diaminobenzidine (50 mL phosphate buffer containing 30 mg DAB and 75 μL 30% H_2O_2). PDC was probed with a rabbit antiserum directed against the *T. glabrata* purified PDC protein (unpublished data).

2.8. Other methods

Protein concentration was determined with the Bradford method [27] using bovine serum albumin (Roche Molecular Biochemicals) as the standard. Basic molecular biology techniques were performed according to the manufactures' instructions or standard procedures [17]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with SDS-Tris system using 10% polyacrylamide gel was carried out according to the procedure described by Laemmli [28] using the Mini-Protean III apparatus (Bio-Rad Laboratories, Inc.).

3. Results

3.1. Isolation and characterization of ura3 mutants of T. glabrata

To generate the *T. glabrata* strain suitable for genetic transformation, strains bearing mutation in the gene (*URA3*) encoding orotidine 5′-phosphate decarboxylase were isolated. The *T. glabrata* IFO005 was used as the parental wild type strain. Stable *ura* mutants were identified after EMS mutagenesis [19] and 5-FOA media selection [18], and purified for single colonies. Since the *ura* phenotypes of *T. glabrata ura* mutant strains could be complemented by the *S. cerevisiae URA3* gene on the episomal plasmid pRUL129, the two *ura3* stains designated as IFO005-3 and IFO005-7 were confirmed. The growth of the parental strain (*T. glabrata* IFO005), *ura3* mutants (*T. glabrata* IFO005-3 and IFO005-7), and *S. cerevisiae URA3* complemented mutants (*T. glabrata* IFO005-36 and IFO005-72) in SD, SD supplemented uracil (20 mg/L), and 5-FOA agars was shown in Fig. 2, all of which gave the evidences of our experiment about screening mutants. Due

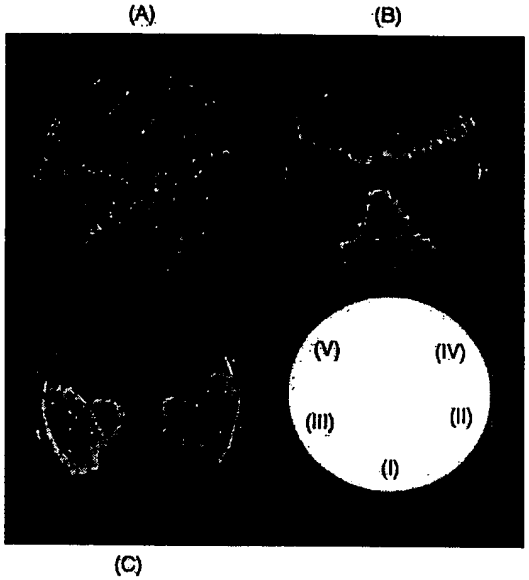

Fig. 2. The growth of *T. glabrata* in SD supplement uracil (200 mg/l) (A), SD (B), and 5-FOA agars (C). (I) *T. glabrata* IFO005; (II) *T. glabrata* IFO005-3; (III) *T. glabrata* IFO005-7; (IV) *T. glabrata* IFO005-36 and (V) *T. glabrata* IFO005-72.

to the *URA3* mutation, strains IFO005-3 and IFO005-7 could grow on 5-FOA agar, but not on SD agar. However, strains IFO005-36 and IFO005-72 complemented by *S. cerevisiae URA3* could grow on SD agar, but not on 5-FOA agar. The parental strain IFO005 could also grow on SD agars, but not on 5-FOA agar because of its normal *UAR3* gene. All strains could grow on SD supplement uracil agar.

By the PCR cloning and sequencing of the *URA3* genes of *T. glabrata* IFO005-3 and IFO005-7 with primers TgURA3-1 and TgURA3-2 (Table 2), we found that some amino acid residues in the *URA3* gene in these two mutants were mutative; two mutations, E185K and T191R, were produced in both mutants. IFO005-3 had the other mutation, C33F and IFO005-7 possessed the other four mutations, K29T, G108R, G128R and P202H. However, all of these mutated amino acids were not conserved [29,30]. We could not tell what gave rise to the *ura3* auxotroph of *T. glabrata* IFO005-3 and IFO005-7, but these mutations should be relevant to *ura3* auxotroph.

3.2. Construction of pdc mutants of T. glabrata

To achieve increased pyruvate production and reduced ethanol production, the most promising and efficient approach was to minimize metabolic flux to the pathways of by-products (ethanol) by metabolic engineering. We disrupt the *PDC* gene in *T. glabrata* to block the pathway from pyruvate to aldehyde, then to ethanol, thus increasing pyruvate accumulation and decreasing ethanol production.

During the construction of *pdc* strains of *T. glabrata, PDC* gene disruption was carried out by homologous recombination (Fig. 1B). The constructed homologous DNA fragment

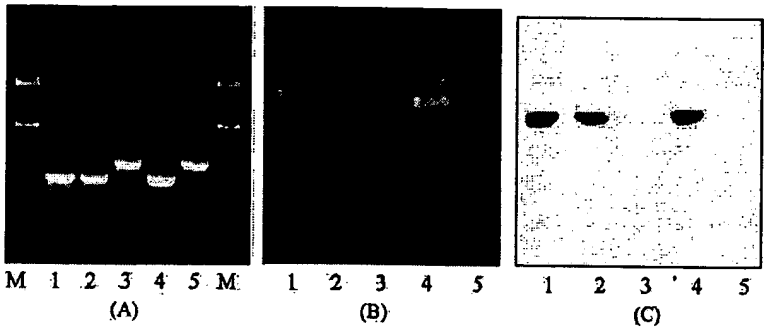

Fig. 3. Characterization of *T. glabrata*. (A) PCR analysis of *T. glabrata*; (B) Pdc activity of crude extract from *T. glabrata*; (C) Western Blot analysis of crude extract from *T.glabrata*. (1) *T. glabrata* IFO005; (2) *T. glabrata* IFO005-3; (3) *T. glabrata* IFO005-36; (4) *T. glabrata* IFO005-7; (5) *T. glabrata* IFO005-72; M) DNA maker (λ DNA/*Eco*RI+*Hin*dIII: 21,227, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 and 125 bp).

(Fragment III) was introduced into *T. glabrata ura3* strains (IFO005-3 and IFO005-7) by lithium acetate transformation, and disruptants appeared after 2–3 days on agar SD medium. *pdc* strains of *T. glabrata* IFO005-36 and IFO005-72 were obtained from IFO005-3 and IFO005-7, respectively.

Based on our laboratory experience, one-step gene disruption in the yeast *T. glabrata* was not straightforward. After a systematic study of several factors that could influence frequency of gene disruption, we found that the length of the target gene region flanking the marker gene is a critical factor in one-step gene disruption in *T. glabrata*. Targeted gene regions of about 500 bp flanking the marker were necessary to obtain a disruption frequency. Targeted gene disruption in some non-conventional yeasts has similar results [31].

3.3. Characterization of pdc strains of T. glabrata

Enzymatic analysis of *pdc* strains, *T. glabrata* IFO005-36 and IFO005-72, grown in YEPD medium demonstrated the presence of very low PDC activity (0.48 and 0.51 U/mg, respectively). Also PDC activities in *T. glabrata* IFO005, IFO005-3, and IFO005-7 were about 1.5–1.7 U/mg. These results indicated that *PDC* gene disruption significantly reduced the enzyme activities of the *pdc* strains. The total DNA isolated from five strains of IFO005 and its derivatives was subjected to PCR analysis using two primers PDC-C31 (forward) and PDC-C32 (reverse) (Table 2). A 2.25-kb fragment was detected in the two disruptants IFO005-36 and 005-72, and a 1.9-kb fragment was detected in wild type strain IFO005, and *ura3* mutants IFO005-3 and 005-7 (Fig. 3A); this was consistent with the disruption of *PDC* as predicted from the yeast genome sequence. Sequencing of their PCR products also detected that the *PDC* gene in parent strains was replaced by homologous DNA fragment (Fragment III), which contained *T. glabrata PDC-F1*, *S. cerevisiae URA3*, and *T. glabrata PDC-F2*. The primer PDC-C31 was in the upstream of PDC starting codon. PDC–activity staining of cell extracts from *T. glabrata* IFO005 and its mutants (Fig. 3B), and Western blot analysis (Fig. 3C) further confirmed *PDC* gene disruption in these mutants. These results showed that PDC-activity and immunologically cross-reacting materials were not detected in the cell extracts from IFO005-36 and IFO005-72. Therefore, *PDC* has been efficiently disrupted in these strains as compared to their parent strains, IFO005-3 and IFO005-7.

3.4. Growth and fermentation pattern of T. glabrata mutants and wild-type strains

Fig. 4 represents the growth on SD medium containing uracil (200 mg/mL) and YEPD medium of IFO005 *pdc* strains with respect to the wild-type and *ura3* strains. From

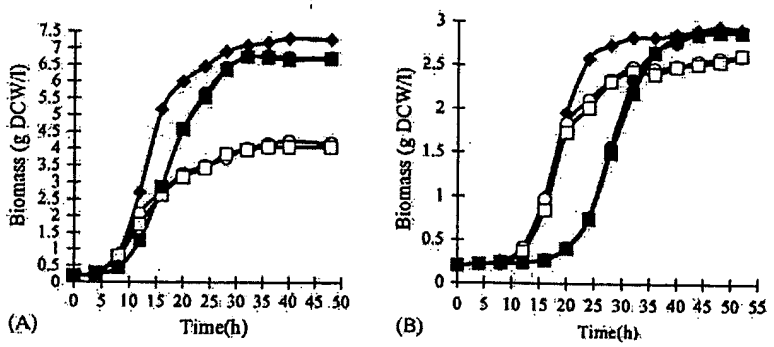

Fig. 4. Consequences *PDC* and *URA3* disruption for growth. (A) YEPD medium; (B) SD medium. (♦) IFO005; (○) IFO005-3;(●) IFO005-36; (□) IFO005-7; (■) IFO005-72.

Table 3
Pyruvate production by *T. glabrata* IFO005 and its derivatives under basal fermentation conditions

Strains	Biomass (g DCW/L)	Pyruvate (g/L)	Residual glucose (g/L)	Ethanol (g/L)
IFO005	18.21 ± 0.66	7.3 ± 0.5	7.6 ± 0.9	7.4 ± 0.4
IFO005-3	7.95 ± 0.39	4.7 ± 0.4	13.6 ± 0.9	7.1 ± 0.3
IFO005-7	7.56 ± 0.36	5.3 ± 0.5	13.1 ± 1.4	6.6 ± 0.4
IFO005-36	16.29 ± 0.36	19.9 ± 0.9	11.3 ± 1.0	4.6 ± 0.4
IFO005-72	15.90 ± 0.51	20.4 ± 1.5	10.2 ± 1.4	4.6 ± 0.4

Scale: 15 mL basal fermentation medium in a 500 mL Erlenmeyer flask, cultivation time (h): 30 h. Data are presented as the average (±) standard deviation for duplicate assays on two independent cultures.

the growth in two media, we found that *pdc* strains have a delay at the early stages of growth, especially in SD medium (Fig. 4B), which showed that *PDC* gene disruption really has an effect on cell growth. The metabolic process of pyruvate by PDC could contribute to NADH/NAD^+ balance [32]. Thus, *PDC* gene disruption would destroy the balance of NADH/NAD^+, which results in the delay of the cell growth of *pdc* mutants. Once NADH/NAD^+ balanced again through the regulation of other metabolic pathway, the cells also restored the growth similar to the wild type strain. As for *ura3* mutants, all growth in various basic media was affected due to the uracil auxotroph (Fig. 4A and B, Table 3).

The afore mentioned *pdc* mutants and their parental strains were isolated from single colonies, and subsequently tested for their ability to produce pyruvate in the basal fermentation medium. The data in Table 3 shows the advantages of *pdc* strains IFO005-36 and IFO005-72 over strains IFO005, IFO005-3, and IFO005-7 in pyruvate production: the former have higher pyruvate production and lower ethanol production in basal fermentation medium. Although IFO005 could grow faster than the *pdc* mutants, its high-biomass did not result in high production of pyruvate. Our previous paper [33] also showed that it was necessary to keep a balance between biomass and pyruvate production during fermentation. Under optimization conditions in 5 L-jar fermentation, the disruptant IFO005-36 accumulated the highest yield of 82.2 g/L of pyruvate in 52 h (Fig. 5), and the yield of pyruvate to glucose was 0.548. As the same time, it only accumulated about 4.7 g/L of ethanol.

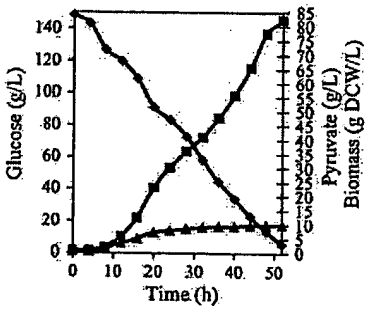

Fig. 5. Pyruvate fermentation by *T. glabrata* IFO005-36 in 5 L-jar fermentor. (■) Pyruvate; (♦) glucose and (▲) biomass.

4. Discussion

T. glabrata is a superior species for pyruvate and optically active α-hydroxyketone production [6,34]. In this investigation, we used EMS mutagenesis and complemented integration to screen the *ura3* auxotrophs. These *ura3* strains of *T. glabrata* should be useful in the metabolic engineering of *T. glabrata* for improved pyruvate or α-hydroxyketone production. *T. glabrata* could also be transformed with circular palsmid carrying ARS elements from *S. cerevisiae* [35] or from other *T. glabrata* strains [36], and the plasmid replicate as unstable, high-copy extrachromosomal element. This was also useful in the metabolic engineering of *T. glabrata* for improved pyruvate or α-hydroxyketone production. The 5-FOA selections were extremely useful for a number of genetic and molecular biological manipulations that required the detection of rare *ura3* cells. The advantages of the 5-FOA selection were the availability of a large collection of *URA3*-based cloning vectors of various types for yeast; the small size and known sequence of the *URA3* gene; the availability of numerous well-studied mutations in the *URA3* gene; and the specificity, case, and efficacy of the selection [18].

The acetate concentration of the fermentation medium had a great effect on pyruvate production by the mutants derived from chemical mutagenesis [15], but not by the disruptants derived from gene disruption (data not shown). These results showed that two species of mutants with reduced PDC activities have difference metabolic flux consequences. The former needed to add acetate to synthesize the adequate acetyl coenzyme A (acetyl-CoA) by acetyl-CoA synthetase (ACS) to reduce the degradation of pyruvate. Thus, more pyruvate could be accumulated after adding acetate during fermentation. The disruptants have no such behavior; consequently there will be a simpler pyruvate production process.

The essential role of PDC in assimilatory carbon metabolism is not a general phenomenon among yeasts. For example, it is an essential enzyme for the growth of *S. cerevisiae* on glucose, even in complex media; mutants of *Kluyveromyces lactis* lacking the unique *PDC1* strain lack pyruvate decarboxylase, but exhibit wild-type specific growth rates on glucose [37]. The role of PDC in *T. glabrata* was apparently different from that of the two yeasts *S. cerevisiae* and *K. lactis* (Fig. 4). However, the detailed mechanism needs to be explored further.

Pyruvate, the end-product of glycolysis, is located at the important branch-point between respiratory and fermentative carbon metabolism. Flux distribution at the pyruvate level is of crucial importance for by-product formation in yeast [32]. During fermentative sugar metabolism, pyruvate is decarboxylated to acetaldehyde by PDC, and the acetaldehyde is subsequently turned into ethanol by alcohol hydrogenase. Dissimilation of pyruvate for respiration requires its conver-

sion to acetyl-CoA, the precursor metabolite of the tricarboxylic acid (TCA) cycle, or to other metabolites of the TCA cycle. It is known that this conversion can occur three ways in *S. cerevisiae*: the direct oxidative pathway of pyruvate to acetyl-CoA via the mitochondrial PDH complex; the indirect oxidative pathway via PDC, acetaldehyde dehydrogenase (A*l*DH) and ACS; and the anaplerotic carboxylation of pyruvate to oxaloacetate catalyzed by PYC [32]. In previous studies [6,15], the degradation of pyruvate was controlled either by a limited addition of thiamine, which is a cofactor of the PDH complex and the PDC enzyme in *T. glabrata*, or by screening mutants with reduced PDC-activity after random mutagenesis. Thiamine-limitation was efficient but not perfect for pyruvate fermentation by *T. glabrata*. Generally, the intracellular pyruvate concentration could be high, but it is soon channeled into ethanol production with increased rates of glycolysis in yeast [38]. In this study, *pdc* mutants derived from *T. glabrata* IFO005 exhibited the ability to produce increased levels of pyruvate than the parent strain (Table 3). This was because the *pdc* mutant had decreased PDC activity; the PDC activities of *pdc* mutants were only about 33% that of the parent strain. It was shown that the reduced PDC activity levels of *pdc* mutants would metabolize less pyruvate to ethanol via acetaldehyde. However, the levels of PDC activity in the parent strains (strains IFO005, IFO005-3, and IFO005-7) were too high to accumulate any significant level of pyruvate, the key intermediate metabolite in both respiration and fermentation. On the other hand, it was presumed that the level of the acetaldehyde-producing enzyme (PDC) and the acetaldehyde-degrading enzymes (ADH and A*l*DH) were well balanced in *pdc* mutants, which had reduced PDC enzyme levels. However, in the parent strain, the balance between these enzymes was not favorable for producing significant levels of pyruvate because its PDC level was too high. These studies demonstrated that it was possible to accumulate high levels of an intermediate metabolite such as pyruvate by altering the level of the PDC enzyme. According to the reported results, an efficient redirecting of metabolic flux to increase pyruvate production requires deletion of by-product synthesis pathways (i.e., ethanol, glycerol, etc.).

Improvement of strains has traditionally relied on random mutagenesis followed by screening for mutants with desirable properties. Now, the opportunity to disrupt related genes and regulatory elements distinguishes metabolic engineering from the traditional genetic approaches. In this work, the use of a straightforward metabolic engineering strategy consisting of *PDC* gene disruption in order to control the excessive conversion of intracellular pyruvate to ethanol increased pyruvate production in the pyruvate-producing yeast, *T. glabrata*. We can attribute the success of this strategy to previous detailed studies of carbon and electron flux [32] which revealed the catabolic behavior of *T. glabrata* with glucose substrates. Our efforts on the metabolic engineering of *T. glabrata* have achieved significant progress in improving the efficiency of the engineered strain in pyruvate production.

Acknowledgments

This work was supported by the National Knowledge Innovation Project from the Chinese Academy of Sciences. We thank Dr. Liu Jiquan, and Dr. H. Yde Steensma for their gifts of strain (*Torulopsis glabrata* IFO005) and plasmid pRUL129, respectively. We thank Dr. Keqian Yang (Institute of Microbiology, CAS) and Dr. Xiaorong Wu (Kansas State University, USA) for revising the manuscript.

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